

## Loss of the Expression of the Tumor Suppressor Gene *ARHI* Is Associated with Progression of Breast Cancer<sup>1</sup>

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### ABSTRACT

**Purpose:** Ductal carcinoma *in situ* (DCIS) is a preinvasive-stage breast carcinogenesis that accounts for ~20~25% of mammographically detected breast cancers. A significant fraction of untreated DCIS will evolve into invasive cancer. *ras homologue 1 (ARHI)* is an imprinted tumor suppressor gene that is expressed in normal breast epithelial cells but absent or down-regulated in breast cancer cells. This study investigated the relationship of *ARHI* expression to the progression of breast cancer.

**Experimental Design:** We analyzed *ARHI* expression in DCIS, invasive breast carcinoma, and adjacent normal breast epithelium from 64 formalin-fixed, paraffin-embedded DCIS specimens by both immunohistochemistry and *in situ* hybridization. We also analyzed the correlation between *ARHI* expression and progression of breast cancer, as well as the correlation of *ARHI* expression and cyclin D1 and p21<sup>WAF1/CIP1</sup> expression in DCIS.

**Results:** Normal breast epithelium was found in all of the specimens and invasive breast carcinoma was found in 23 specimens. *ARHI* mRNA and protein were detected in all of the normal breast epithelia. *ARHI* expression was detected mainly in cytoplasm and rarely present in the nucleus. By histochemical analysis, *ARHI* expression was down-regulated in 41% (26 of 64) of DCIS and 70% (16 of 23) of invasive carcinomas comparing the specimens with adjacent normal breast epithelium. When DCIS and invasive cancer were present in the same sample, *ARHI* was further down-

regulated in 26% (6 of 23) of invasive carcinoma. In four cases [4 (17%) of 23] of invasive carcinoma, *ARHI* protein expression was totally lost. Consistent results were obtained with an *in situ* hybridization assay for *ARHI* at the mRNA level. Higher levels of expression of cyclin D1 and p21<sup>WAF1/CIP1</sup> were observed in DCIS than in the adjacent epithelia. The expression of cyclin D1 and p21<sup>WAF1/CIP1</sup> was inversely correlated with that of *ARHI*.

**Conclusions:** Our results indicate that *ARHI* expression is markedly down-regulated in DCIS, and a further decrease in *ARHI* expression is associated with progression of breast cancer.

### INTRODUCTION

The natural history of breast cancer involves a sequential progression through defined clinical and pathological stages starting with atypical hyperproliferation, progressing into *in situ*, then invasive carcinomas, and culminating in metastatic disease. It has been estimated that more than 46,000 patients would be diagnosed with DCIS<sup>3</sup> of the breast in 2001, representing 19% of all newly diagnosed breast cancer cases (1). DCIS refers to a heterogeneous group of noninvasive breast cancers, and the proper treatment of these lesions remains controversial. A significant fraction of untreated DCIS will evolve into invasive cancer.

*ARHI* is an imprinted tumor suppressor gene that is expressed in normal breast epithelial cells but is down-regulated in breast cancer cells (2). *ARHI* is a member of the *ras* superfamily sharing 54~62% amino acid homology with *ras* and *rap*. The function of *ARHI*, however, differs from that of the *ras* and *rap* proteins. *ARHI* has been mapped to a region of chromosome 1p31 that has been found deleted in a substantial fraction of ovarian and breast cancers. The retained allele has been found to be imprinted, methylated, and silenced in 7 of 9 informative cases (3), consistent with a loss of expression through imprinting and a LOH of the nonimprinted allele. To the extent that the association of growth regulatory genes with LOH has signaled the presence of tumor suppressor loci, *ARHI* is a putative tumor suppressor gene in these malignancies. Introduction of *ARHI* can inhibit the growth of transformed cells that have lost expression of the endogenous gene. The role of *ARHI* may depend on the cellular context in which the protein is expressed.

To investigate the relationship of *ARHI* expression to the progression of breast cancer, we examined *ARHI* expression in

Received 10/18/02; revised 3/31/03; accepted 4/12/03.

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<sup>1</sup> Supported by NTH Grants CA64602, CA80957, and N01-CN-65004 and by a grant from the Susan G. Komen Breast Cancer Foundation.

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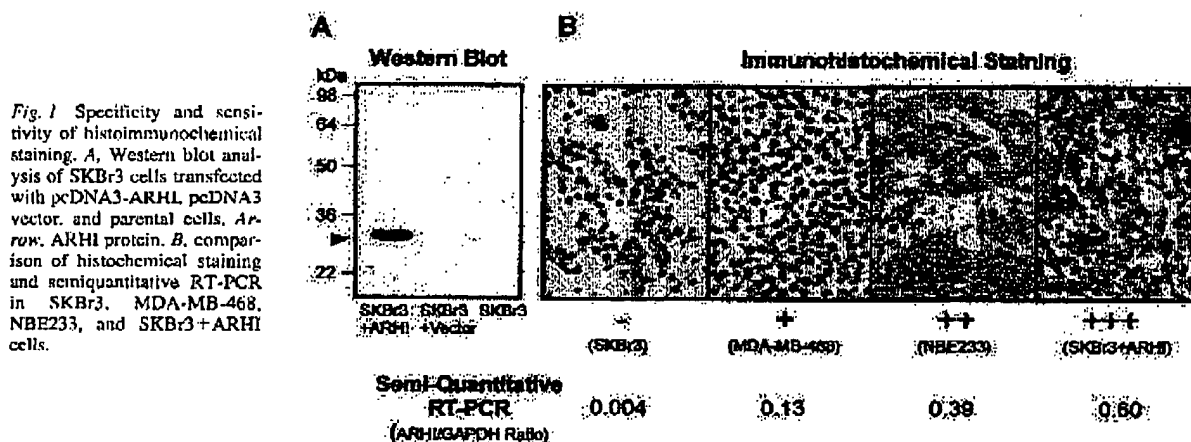
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<sup>5</sup> The abbreviations used are: DCIS, ductal carcinoma *in situ*; *ARHI*, *ras homologue member 1*; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RXR- $\alpha$ , retinoid X receptor- $\alpha$ ; ISH, *in situ* hybridization; IHC, immunohistochemistry; LOH, loss of heterozygosity; GST, glutathione S-transferase; NSS, normal sheep serum; RT-PCR, reverse transcription-PCR; NBE, normal breast epithelium/epithelia.

EXHIBIT

A



**Fig. 1** Specificity and sensitivity of histoimmunohistochemical staining. **A**, Western blot analysis of SKBr3 cells transfected with pcDNA3-ARHI, pcDNA3 vector, and parental cells. Arrow, ARHI protein. **B**, comparison of histochemical staining and semiquantitative RT-PCR in SKBr3, MDA-MB-468, NBE233, and SKBr3+ARHI cells.

64 formalin-fixed, paraffin-embedded DCIS specimens from the Breast Cancer Tissue Bank at M. D. Anderson Cancer Center. We found that ARHI mRNA and protein were identified in all of the NBE. ARHI expression was down-regulated in DCIS and further down-regulated in invasive carcinomas.

## MATERIALS AND METHODS

**Identification of Patient Material.** Paraffin-embedded archival breast tissue specimens and surgical pathology reports for 64 consecutively examined DCIS cases with or without an invasive component were identified from the pathology database of The University of Texas M. D. Anderson Cancer Center during the period of 1986–1999. Twenty-three of 64 DCIS specimens contained an invasive component in the same slide. An additional three paraffin-embedded normal breast specimens were obtained from the department of Plastic Surgery. H&E-stained slides of each DCIS case were reviewed and graded by a breast pathologist (A. A. S.) using standard grading criteria, as described previously (1). Sociodemographic characteristics and clinical variables were abstracted from patients' medical records.

**Prepared ARHI Monoclonal Antibody.** An ARHI cDNA fragment which included all four GTP binding domains has been obtained by PCR amplification and fused in-frame into pGEX-2T vectors to produce recombinant constructs GST-ARHI that expressed a  $M_r$  53,000 fusion protein of which  $M_r$  27,000 was GST and  $M_r$  26,000 was ARHI derived. Large quantities of fusion protein were prepared and purified by preparative SDS-PAGE. Rabbit antiserum and murine monoclonal antibodies were prepared by standard protocols. Seven monoclonal antibodies against GST-ARHI were obtained and used for detecting ARHI protein.

ARHI has also been cloned into bacterial systems for expressing 6-His-tagged protein. Soluble and functional full-length human ARHI was purified by Ni-NTA (Qiagen, Valencia, CA) affinity chromatography followed by other chromatographic techniques. We had been able to purify the recombinant pARHI and used the protein to generate highly specific mono-

clonal antibodies.<sup>6</sup> All of the monoclonal and polyclonal antibodies that reacted against ARHI were screened in tissue sections; the most specific monoclonal antibody 15E11 (IgG1) was used in this study. Western blot using this antibody was used to analyze the specificity of this antibody, a single band of  $M_r$  26,000 protein was shown in ARHI-transfected breast cancer cells but not in parental cells (Fig. 1A).

**IHC.** Staining was performed on 3- $\mu$ m sections of formalin-fixed, paraffin-embedded tissue that were rehydrated through graded ethanol. Antigen retrieval was performed by heating the sections 3 min under pressure in a pressure cooker in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase activity was quenched by incubating sections in 3% hydrogen peroxide. Slides were incubated in Ultra V Block for 5 min at room temperature to block nonspecific staining. The ARHI monoclonal antibody (IgG1) was applied overnight at 1.16  $\mu$ g/ml in 1% BSA-PBS at 4°C overnight. Biotinylated secondary antibodies were detected with streptavidin peroxidase by using the UltraVision Large Detection System Anti-Polyvalent, HRP (Lab Vision Corporation, Fremont, CA). A diaminobenzidine tetrachloride supersensitive substrate kit (Biogenex, San Ramon, CA) was used to visualize the antibody-antigen complex. After a final wash with distilled water, light counterstaining was performed with hematoxylin. Sections of normal breast NBE served as a positive control. Two negative controls were carried out in all samples, ARHI antibody was replaced by PBS and leukocyte common antigen (LCA) monoclonal antibody (IgG1).

Immunohistochemical staining of cyclin D1 and p21<sup>WAF1/CIP1</sup> (antibodies were purchased from Neomarkers, Fremont, CA) was performed following the protocol provided by the manufacturer. Breast cancer and colon cancer sections provided by the manufacturer were used as the positive controls for cyclin D1 and p21<sup>WAF1/CIP1</sup>, respectively. Methods for

<sup>6</sup>R. Z. Luo and Y. Yu, unpublished observations.

Ki-67 staining were described previously (4). Antibody for detection of Ki-67 was purchased from Dako (Carpinteria, CA).

**Preparation of Digoxigenin-labeled RNA Probe.** The ARHI cDNA (1012 bp), which spanned the entire open reading frames, was subcloned into plasmids in orientation with respect to either the Sp6 or the T7 promoter site to transcribe antisense or sense RNA probes. The plasmids were linearized by *Small* digestion and were purified by phenol-chloroform-isoamyl alcohol extraction. A transcription kit (Roche, Mannheim, Germany) was used to transcribe digoxigenin-labeled cRNA probes. Linearized DNA (1 µg/ml) was incubated for 2 h at 37°C in a solution containing transcription buffer, nucleotide triphosphate-labeling mixture, Sp6 or T7 RNA polymerase, and RNase inhibitor. The reaction was stopped by digesting the DNA template with RNase-free DNase. The RNA probes were precipitated with LiCl and 100% ice-cold ethanol overnight at -70°C, then pelleted by centrifugation at 12,000 rpm at 4°C, and further washed with 70% ethanol. The probes were then dried under vacuum and dissolved in 0.1 M diethyl pyrocarbonate water containing 2 mM EDTA, and their concentration was adjusted to 100 ng/µl. The digoxigenin-labeled RNA probes were stored at -80°C.

**ISH.** ISH was carried out following the procedure described by Xu *et al.* (5). Briefly, the sections were deparaffinized, rehydrated, and deproteinized. The slides were then prehybridized at 42°C for 1 h in Slide Mount Model 240000 (Boeckel Scientific, Feasterville, PA) and incubated (50 µl/slide) with hybridization solution containing freshly denatured digoxigenin-labeled cRNA probe (0.625 µg/ml) at 42°C for 4 h. The sections were washed twice in 2× SSC and further washed in 2× SSC containing 0.2% NSS and 0.5% Triton X-100 for 2 h with mild agitation. For the immunodetection of the ISH signal, the slides were incubated in buffer 1 [0.1 M maleic acid-0.15 M NaCl (pH 7.5)] containing 0.2% NSS and 0.3% Triton X-100 for 30 min at room temperature, and were further incubated overnight at 4°C with sheep anti-digoxigenin antibody (0.75 µg/ml in buffer 1 containing 1% NSS and 0.3% Triton X-100). The slides were then washed twice in buffer 1 and then with buffer 2, which consisted of 0.1 M Tris-0.1 M NaCl-MgCl<sub>2</sub> (pH 9.5). The color reactions were developed by incubating the slides in a chromogen solution (45 µl of nitroblue tetrazolium and 35 µl of X-phosphate solution in 10 ml of buffer 2) in humidified light-tight containers for up to 6 h, with observation for color development. The color reaction was stopped by washing the slides with Tris-EDTA buffer, and the slides were mounted with a cover glass in Aqua mounting medium.

The specificity of the method was confirmed by the negative results obtained with corresponding sense controls, and the distinct positive staining patterns obtained with digoxigenin-labeled GAPDH mRNAs and antisense probes (Tetra Link, Buffalo, NY) and digoxigenin-labeled RXR-α mRNA antisense probes, which were a kind gift from Dr. Xu (University of Texas M. D. Anderson Cancer Center, Houston, TX) (6).

**Semiquantitative RT-PCR.** Total RNA was extracted from different cells by the Trizol method (Invitrogen, Carlsbad, CA). cDNA was synthesized using 2 µg of total RNA. Oligo(dT)<sub>18</sub> and SuperScript II reverse transcriptase (Invitrogen,

Carlsbad, CA) were used for the reverse transcriptase reaction according to the manufacturer's instructions. Semiquantitative RT-PCR was performed in Agilent 2100 Bioanalyzer (Germantown, MD) according to the instructions of the manufacturer with two gene-specific primers: NY2P1, 5'-TCTCTCCGAG-CAGCGCA-3', and 3Y2SP2, 5'-CGTCGCCACTCTTGCT-GTCG-3'. The amount of ARHI expression was normalized to a human GAPDH endogenous reference standard and was presented as the ratio of ARHI:GAPDH.

**Transfection and Western Blot Analysis.** Breast cancer cell line SKBr3 was transfected with pcDNA3 vector only or with pcDNA3-ARHI as described previously (2). Cell lysates were analyzed by Western blot as described previously (2), using the same antibody 15E11 as in the immunohistochemical staining.

**Semiquantitative Estimation for Immunohistochemical and ISH Staining Results.** The cytoplasmic intensity and proportion of positive cells for ARHI IHC and ISH staining were determined for the NBE, the DCIS, and the invasive carcinoma. On the basis of criteria used by similar publications (7), we prospectively chose 10% as a cutoff for positive-staining cells and used a subjective scale (- to +++) to classify staining patterns, as follows: (a) -, no staining; (b) +, weak staining; (c) ++, moderate staining; and (d) +++, intense staining. If (c) or (d) was evident, the specimen was considered to be strongly positive. We examined a subset of four cell lines with different expression level [breast cancer cell lines SKBr3 and MDA-MB-468, NBE 233, and SKBr3 transfected with pcDNA3-ARHI (SKBr3+ARHI)] by immunohistochemical staining and semiquantitative RT-PCR. Four cell lines were fixed in 90% ethanol. The pellets were embedded in paraffin and stained as described above. Fig. 1B shows the correlation of immunohistochemical staining intensity and ARHI expression levels by semiquantitative RT-PCR. For cyclin D1, p21<sup>WAF1/CIP1</sup>, and Ki-67, yellow or brown nuclear staining was considered positive. Faint nuclear staining or cytoplasmic staining was not considered significant. At least 500 cells were counted in high-staining density area in every component on every slide. Two % positive was used as the cutoff level for cyclin D1 and p21<sup>WAF1/CIP1</sup> (8, 9), and 3% positive for Ki-67, as suggested by Mommers *et al.* (9).

**Statistical Analysis.** We recorded labeling indices of ARHI as 0 (no expression), 1 (weak expression), 2 (moderate expression), and 3 (strong expression) for statistical analyses. Labeling indices of cyclin D1, p21<sup>WAF1/CIP1</sup>, and Ki-67 were recorded as a continuous variable. We used Wilcoxon signed-rank tests to compare ARHI expression in DCIS, invasive ductal carcinoma, and adjacent normal breast tissue. The McNemar  $\chi^2$  test was used to compare discordance between ARHI immunostaining and ISH. Paired *t* tests were used to compare mean labeling indices of cyclin D1, p21<sup>WAF1/CIP1</sup>, and Ki-67 between adjacent normal mammary tissue and DCIS and invasive ductal carcinoma in the same slide. All of the statistical computations were performed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, Illinois) and STATISTICA computer software programs (StatSoft, Inc., Tulsa, Oklahoma). A *P* below 0.05 was considered statistically significant.

Table 1 Patient characteristics

Characteristics	DCIS (n = 64)
Median age at diagnosis (range)	47 yr (27-74 yr)
Race	
White	44
Black	6
Hispanic	8
Asian	5
Unknown	1
Median age at menarche (range)	13 yr (10-16 yr)
Median age at first childbirth (range)	23 yr (15-38 yr)
Nuclear grade	
Grade 1	2
Grade 2	24
Grade 3	38
Necrosis	
Absent	22
Focal	19
Extensive	23

## RESULTS

**Patient Characteristics.** The characteristics of the patients whose specimens were analyzed are shown in Table 1. White women constituted a majority. The median age at diagnosis, median age at menarche, and the median age at first childbirth were 47, 13, and 23 years, respectively. There were 2 grade-1, 24 grade-2, and 38 grade-3 DCIS lesions. Almost two-thirds of the DCIS cases had either focal or extensive necrosis.

**ARHI Expression in NBE, Adjacent Normal Mammary Epithelia, DCIS, and Invasive Carcinoma.** ARHI expression was concentrated in cytoplasm diffusely and rarely presented in the nucleus. In some cases, positive immunohistochemical staining was found around part of the nucleus, as in the area of the Golgi body in the cytoplasm. By immunohistochemical analysis, all duct and lobular epithelia in three normal breast tissue specimens showed strong ARHI expression. Similarly, ARHI expression in adjacent normal epithelia obtained from cancer patients showed strong positivity in 59 (92%) of 64 cases, and weak positivity in other 5 (8%) cases. No cases lacked ARHI expression in adjacent normal epithelial cells (Fig. 2; Table 2). In contrast, ARHI expression was decreased in both DCIS and the invasive components. Thirty-nine of 64 (61%) specimens showed strong expression of ARHI in DCIS, and 9 (39%) of 23 showed strong expression in the invasive cancer component.

**Down-Regulation of ARHI Expression in DCIS and Invasive Carcinoma.** We observed a statistically significant reduction of ARHI expression in DCIS when compared with adjacent NBE ( $P < 0.00001$ ; Table 3). Twenty-six (41%) of 64 cases showed decreased expression of ARHI in DCIS compared with adjacent normal epithelia. Thirty-eight specimens (59%) showed a similar level of expression both in DCIS and adjacent normal epithelia. None of the DCIS components showed stronger ARHI expression than that found in adjacent normal epithelia. A similar pattern was observed when ARHI expression was compared between invasive components and adjacent normal epithelia. Of the 23 specimens that contained both DCIS and

invasive components in the same slide, we observed further down-regulation of ARHI expression in the invasive carcinoma component compared with adjacent normal epithelia ( $P < 0.000001$ ; Table 3). Sixteen (70%) of 23 specimens showed decreased expression of ARHI. Only 7 (30%) of 23 specimens showed similar intensity of ARHI staining in both invasive cancer and adjacent normal epithelia. None of the invasive components showed stronger expression of ARHI than was found in adjacent normal epithelia. We also compared ARHI expression between DCIS and the invasive component in the same slide and found a statistically significant decrease in DCIS ( $P < 0.02$ ). Six (26%) of 23 specimens showed decreased expression of ARHI in invasive cancer compared with adjacent DCIS. Seventeen specimens (74%) showed similar staining intensity in both invasive and DCIS component. Again, none of the invasive component showed stronger ARHI expression than that was found in DCIS. Fig. 2 demonstrates that ARHI was highly expressed in the adjacent normal epithelia and down-regulated in DCIS lesions, and was further down-regulated in invasive cancer in a specimen from one patient that contained adjacent normal epithelia, DCIS, and invasive cancer.

### Immunohistochemical Method Is Consistent with ISH.

To confirm the pattern of ARHI expression in DCIS, we performed ISH in 64 cases using an ARHI-specific antisense probe, which resulted in a dark purple color in the cytoplasm, in which mRNA is expected to be localized. An ARHI mRNA sense probe was used as a negative control and antisense probes to GAPDH and RXR- $\alpha$  were used as positive controls. Fifty-two cases bound GAPDH and RXR- $\alpha$  antisense probes, indicating that the mRNA of the 52 cases was undegraded. These 52 cases were used for ISH analysis. The ARHI sense probes did not show positive staining, indicating that the hybridization of the antisense probe was specific. Positive ARHI staining was also present on lymphocytes, blood vessels, fibroblasts, and stromal cells. ISH analysis in these 52 DCIS cases revealed that ARHI was down-regulated at the level of mRNA in 22 (42% of 52). In the case depicted in Fig. 2, very similar ISH and IHC analysis results were observed. We compared discordance between ARHI immunostaining and ISH and found high concordance between them ( $P > 0.75$ ), consistent with the regulation of ARHI at the level of transcription.

**Relationship of Cyclin D1, p21<sup>WAF1/CIP1</sup>, and Ki-67 Overexpression to ARHI Down-Regulation in DCIS.** To understand the role of ARHI in the proliferation and progression of DCIS, we also evaluated the expression of cyclin D1, Ki-67, and p21<sup>WAF1/CIP1</sup> in the same specimens. Expression of all three markers was increased in DCIS when compared with adjacent normal epithelia. In Fig. 3, strong expression of cyclin D1, p21<sup>WAF1/CIP1</sup>, and Ki-67 was found in a DCIS lesion, with very weak expression in normal epithelia in a case in which ARHI was down-regulated. Relationships between cyclin D1, p21<sup>WAF1/CIP1</sup>, and Ki-67 and ARHI expression are summarized in Table 4. In the ARHI down-regulation group, mean labeling indices for cyclin D1 ( $P < 0.000001$ ), Ki-67 ( $P < 0.000001$ ) and p21<sup>WAF1/CIP1</sup> ( $P < 0.006$ ) were all significantly higher in the DCIS than in adjacent normal epithelia. In the group with normal ARHI expression, mean labeling indices of both cyclin D1 ( $P < 0.001$ ) and Ki-67 ( $P < 0.000001$ ) were also higher in DCIS lesions than in adjacent normal mammary epithelia.

## 3664 ARHI Expression in DCIS of the Breast

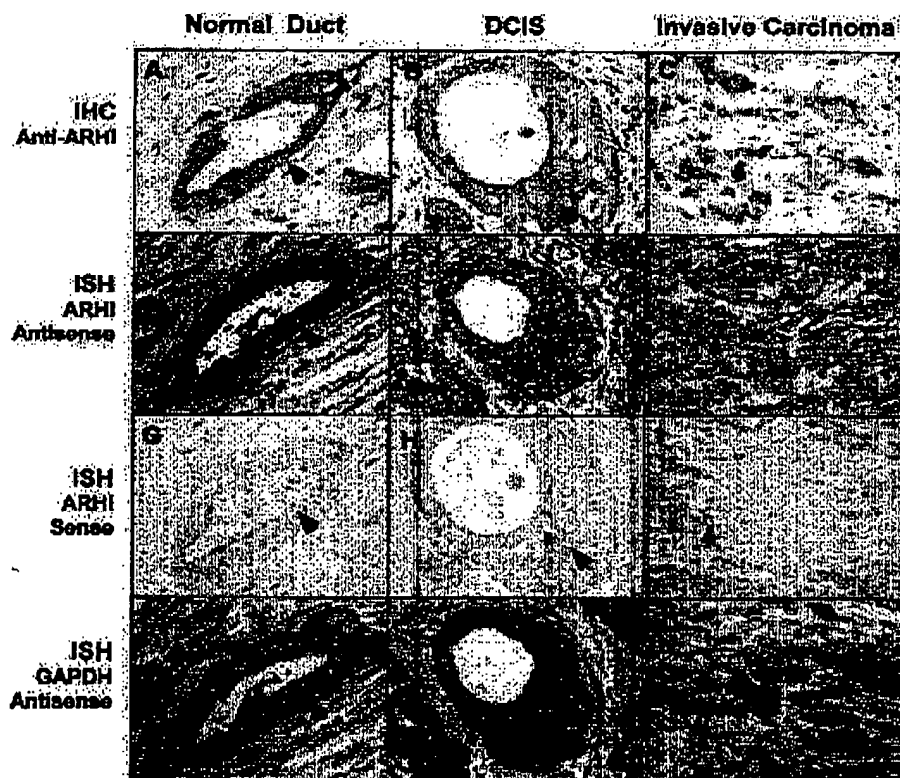


Fig. 2 Down-regulation of ARHI in DCIS and invasive breast cancer. IHC (using an anti-ARHI monoclonal antibody) and ISH (using an ARHI antisense probe) were performed in a case in which consecutive histological sections were from a single tissue block that included normal duct, DCIS, and invasive carcinoma. An ARHI sense probe was used as a negative control; a GAPDH antisense probe was used as a mRNA quality control. The figures show that the expression of ARHI mRNA is strongly positive in adjacent normal duct and is progressively lost in DCIS and invasive cancer. IHC and ISH showed concordant change. Arrowheads, the corresponding locations of ARHI expression in normal epithelium and cancers from different staining methods.  $\times 400$ .

Table 2 ARHI expression in adjacent normal tissue, DCIS, and invasive breast cancer

ARHI	Adjacent normal tissue (n = 64)		DCIS (n = 64)		Invasive breast cancer (n = 23)	
	n	%	n	%	n	%
Negative (-)	0		2	3	4	17
Positive (+)	5	8	23	36	10	44
Positive (++)	39	61	29	45	6	26
Positive (+++)	20	31	10	16	3	13

p21<sup>WAF1/CIP1</sup> was up-regulated in this group but the differences did not achieve statistical significance. Increased expression of cyclin D1 and p21<sup>WAF1/CIP1</sup> in DCIS is not restricted to the ARHI down-regulated group. Table 4 shows that there is a 3-fold increase of cyclin D1 and a 2.8-fold increase of p21<sup>WAF1/CIP1</sup> expression in DCIS (compared with adjacent tissue) among the ARHI-down-regulated group, and only a 1.6-fold increase of cyclin D1 and a 1.8-fold increase of p21 expression in the group that retains ARHI expression. Mean labeling indices of Ki-67 were higher in DCIS lesions than in adjacent normal mammary tissue, but no correlation between ARHI expression status and elevated Ki-67 expression was found. Furthermore, no significant differences in cyclin D1,

Table 3 Comparison of ARHI expression in DCIS, invasive cancer, and adjacent normal breast tissue

ARHI expression	No. of specimens	Mean rank	P <sup>a</sup>
DCIS vs. adjacent normal tissue			
ARHI Expression ↓ <sup>b</sup>	26	13.5	<0.000001
ARHI Expression ↑	0	0	
ARHI Expression =	38		
Total	64		
Invasive cancer vs. adjacent normal tissue			
ARHI Expression ↓	16	8.5	<0.000001
ARHI Expression ↑	0	0	
ARHI Expression =	7		
Total	23		
Invasive cancer vs. DCIS			
ARHI Expression ↓	6	3.5	<0.02
ARHI Expression ↑	0	0	
ARHI Expression =	17		
Total	23		

<sup>a</sup> P based on Wilcoxon signed-rank test.

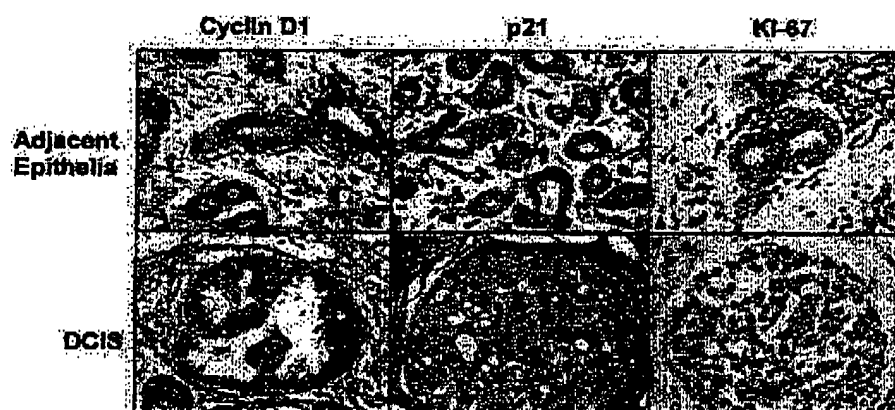
<sup>b</sup> ↓, decreased; ↑, increased; =, similar.

Ki-67, and p21<sup>WAF1/CIP1</sup> expression were found between DCIS and invasive carcinoma.

## DISCUSSION

The development of breast cancer is a process that not only involves up-regulation of proliferation factors and activation of

**Fig. 3** Overexpression of cyclin D1, p21<sup>WAF1/CIP1</sup>, and Ki-67 in DCIS by immunohistochemical staining. Anti-cyclin D1, anti-p21<sup>WAF1/CIP1</sup>, and anti-Ki-67 monoclonal antibodies were used to detect the levels of the three proteins in a case that included adjacent normal duct and DCIS in the same tissue section. In this case, ARHI was down-regulated in DCIS.  $\times 400$ .



**Table 4** Cyclin D1, Ki67, and p21 expression in DCIS, invasive cancer, and adjacent normal breast tissue stratified by ARHI expression

Biomarker	Mean labeling index (%)	SE	P
ARHI down-regulated			
Cyclin D1			
Adjacent normal (n = 24)	11.55	2.65	<0.000001
DCIS (n = 24)	35.67	4.53	
Ki67			
Adjacent normal (n = 24)	1.42	0.40	<0.000001
DCIS (n = 24)	6.44	1.03	
p21			
Adjacent normal (n = 25)	3.95	1.78	<0.006
DCIS (n = 25)	11.26	2.65	
ARHI expressed			
Cyclin D1			
Adjacent normal (n = 37)	17.88	2.62	<0.001
DCIS (n = 37)	29.34	3.13	
Ki67			
Adjacent normal (n = 37)	1.02	0.16	<0.000001
DCIS (n = 37)	7.36	1.08	
p21			
Adjacent normal (n = 37)	4.14	1.13	>0.16
DCIS (n = 37)	7.75	2.39	

oncogenes but also dysregulation of inhibitory factors and loss of tumor suppressor gene function. Early aberrations in proliferation probably only slightly perturb the pathways activated by systemic hormones (estrogen and progesterone) and local growth factors (such as tumor growth factor- $\alpha$  and insulin-like growth factor 2). Telomerase overexpression has been detected in the earliest stages of breast cancer and likely contributes to the immortalization of breast cancer cells (10). The next steps in tumorigenesis involve spontaneous gene amplification (such as *HER-2* and *cyclin D1*), LOH, and mutations (TP53) that promote genomic and chromosomal instability. Although Ras is not often mutated in breast cancer, physiological activation of Ras is frequently associated with malignant progression (11).

DCIS of the breast is a preinvasive stage of carcinoma in the process of multistep breast carcinogenesis that now accounts for ~20–25% of mammographically detected breast cancers

(12). DCIS is a biologically and morphologically heterogeneous disease. If left untreated, a significant fraction of these tumors will evolve into invasive cancer. The molecular mechanisms of DCIS carcinogenesis include dysregulation of the cell cycle and changes of the apoptotic threshold.

Several biomarkers, such as Ki-67 and cyclin D1, have been reported to correlate with the progression of breast cancer from DCIS to invasive carcinoma (4, 9). Here we report a new biomarker ARHI, the expression of which is associated with progression of breast cancer. ARHI is a novel tumor suppressor gene in breast cancer, it encodes a small GTP-binding protein that belongs to the Ras/Rap superfamily, but that has the characteristics of a tumor suppressor gene despite 54–59% homology to the Ras proto-oncogenes. ARHI re-expression blocks heregulin stimulation, truncates signaling through mitogen-activated protein (MAP) kinase, activates JNK, induces p21<sup>WAF1/CIP1</sup>, and down-regulates cyclin D1 promoter activity (Ref. 2 and Yu *et al.*).<sup>7</sup> Hisatomi *et al.* (13) reported ARHI expression was lost or markedly reduced in 12 (46%) of 26 breast cancer tissue samples. Although their study has shown the importance of ARHI inactivation in breast tumor pathogenesis, the technique they used is real-time PCR, in which the expression level in tumor tissue may mix with normal tissue. In this study, both IHC and ISH were used to evaluate ARHI expression in breast cancer tissues which contain adjacent normal tissue, DCIS, and invasive cancer in the same section. Consistent results were obtained from both techniques. ARHI is expressed in normal breast epithelial cells but is dramatically down-regulated in more than 60% of breast cancers. Loss of ARHI expression has been linked to tumor progression from *in situ* to invasive disease.

The cell cycle-regulatory gene, cyclin D1, plays a critical role in the growth and progression of several types of human cancer, including breast cancer. Several studies have shown that cyclin D1 is frequently overexpressed in human breast DCIS specimens (9, 13), which confers a high risk for the develop-

<sup>7</sup> Y. Yu, R. Z. Lun, and X. Fang, unpublished observations.

ment of infiltrating ductal carcinoma. In this study, we compared cyclin D1 and ARHI expression in DCIS samples. We found cyclin D1 level was higher in DCIS lesions than in adjacent normal mammary epithelia in more than 80% cases. Interestingly, however, mean labeling indices of cyclin D1 were much higher in DCIS lesions than in adjacent normal mammary epithelia in the ARHI down-regulated group, as compared with the ARHI normal expression group; this indicated that ARHI down-regulation did correlate with enhanced cyclin D1 expression in DCIS, which is in contrast to expectations based on the effect of ARHI on cyclin D1 promoter activity in cell culture (2). In the *in situ* lesions, cyclin D1 may be amplified at the genomic level and no longer susceptible to transcriptional regulation by ARHI.

p21<sup>WAF1/CIP1</sup>, an inhibitor of cyclin-dependent kinases, is a critical downstream effector in the p53-specific pathway of growth control. p21<sup>WAF1/CIP1</sup> can also be induced by p53-independent pathways during terminal differentiation. We have reported that re-expression of ARHI induces p21<sup>WAF1/CIP1</sup> (2). In this study, we examined the expression level of p21<sup>WAF1/CIP1</sup> in DCIS samples. We observed that the p21<sup>WAF1/CIP1</sup> level was increased in 41% of DCIS cases when compared with adjacent normal tissues. In the remaining 59% of DCIS cases, p21<sup>WAF1/CIP1</sup> level either was down-regulated (15%) or was not significantly different (44%). Significant increases of the expression of p21<sup>WAF1/CIP1</sup> in DCIS were observed in cases with decreased ARHI expression ( $P < 0.006$ ) but not in the cases with normal ARHI expression ( $P > 0.16$ ). There are several reports of p21<sup>WAF1/CIP1</sup> histochemical staining in DCIS. Although the differences in the reported frequency may reflect the differences in the antibodies used, the immunohistochemical methods, and the positive cutoff points, p21<sup>WAF1/CIP1</sup> overexpression in DCIS is common. Mommers *et al.* (8) had shown 49–50% overexpression of p21<sup>WAF1/CIP1</sup> in DCIS and 17–31% in invasive cancer. Oh *et al.* (14) found that 33 (67.3%) of the 49 DCIS cases were positive for p21<sup>WAF1/CIP1</sup> protein. p21<sup>WAF1/CIP1</sup> expression was significantly related to well-differentiated histological grade, negative p53, and the presence of estrogen receptor. Barbareschi *et al.* (15) found high expression of p21<sup>WAF1/CIP1</sup> in DCIS and invasive breast cancer. Rey *et al.* (16) found that p21<sup>WAF1/CIP1</sup> is associated with cyclin D1 expression, suggesting that p21<sup>WAF1/CIP1</sup> overexpression is an early event in breast carcinogenesis that precedes stromal infiltration. Our observations in this study are similar to these reports. The correlation of ARHI and p21<sup>WAF1/CIP1</sup> in DCIS and breast cancer needs to be addressed further in larger studies that take into account the functional status of p53, one of the inducers of p21<sup>WAF1/CIP1</sup>.

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